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Current Thinking

Vitamin E and selenium participation in fatty acid desaturation A proposal for an enzymatic function of these nutrients

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Summary

A critical review of the literature on the effects of vitamin E and selenium deficiences on unsaturated fatty acid metabolism reveals that some of these effects are inconsistent with the antioxidant hypothesis of these nutrients as their only biological function. On the basis of these data it is proposed that vitamin E and selenium play a role in the desaturation of n-3 and n-6 polyunsaturated fatty acids by participating in the microsomal electron transport chain and in a proposed peroxidase moiety of the desaturase complex, respectively. A re-interpretation of the experimental literature in terms of the proposed hypothesis is provided, with some suggestions to test its main tenets.

Introduction

In spite of the fact that vitamin E was discovered more than sixty years ago (1) as the factor that prevented fetal death and resorption in the rat, the molecular mechanism of its biological action remains uncertain (2–4). Although the antioxidant hypothesis proposed by Dam (5) (and later espoused by Tappel (6)) has been challenged (7–13) it has dominated the experimental work on the possible molecular role of tocopherols (for a review see ref. 14). This hypothesis, however, has not been formulated in a manner that proposes testable and necessary tenets and corollaries. In fact, most of the explicit proposed tenets of the proposed hypothesis have been postulated by some of its challengers rather than by its proponents. Therefore, much of the evidence in its support is only vaguely theory driven and thus has seldom been subjected to a rigorous analysis of its presumed tenets.

As suggested initially by MacKenzie (15), vitamin E may have a free-radical scavenger role as well as a more specific enzymatic function *in vivo*. Evidence is accumulating that suggests that vitamin E does have an enzymatic role. As discussed by McCay and King (3), the specific requirement of RRR-alpha-tocopherol (alpha-d-tocopherol) for the phenotypic differentiation of the rotifer (16) is unlikely to be explained by the antioxidant hypothesis. The selective sensory axonopathy of large caliber myelinated fibers produced in Rhesus monkeys by vitamin E deficiency (17) is also unlikely to be caused just by peroxidation. Similarly, the effects of vitamin E on differentiation of neuroblastoma cells (18) and metamorphosis of various insect species (19) are also more likely to be due to a vitamin or growth factor-like effect. Similar conclusions can be made for the effects of selenium and vitamin E on growth and polyunsaturated fatty acid synthesis in cultured mouse fibroblasts (20, 21). These effects could not be reproduced by artificial antioxidants.

This paper critically reviews the experimental literature on the effects of vitamin E and selenium

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deficiencies, with emphasis on polyunsaturated fatty acid metabolism, and concludes that the data cannot be adequately explained by the proposed antioxidant roles of these nutrients. A new enzymatic role for vitamin E and selenium, which is consistent with the literature data, is proposed.

2. Evidence incompatible with the antioxidant hypothesis

2.1. Effects of n-3 fatty acids on encephalomalacia and muscular dystrophy

One of the necessary corollaries of the antioxidant hypothesis is that a condition that is produced by vitamin E deficiency in the presence of dietary C18:2n-6 should have a higher incidence when a more unsaturated fatty acid, such as C18:3n-3, is provided in the diet. This is expected since the rate of peroxidation increases drastically with degree of unsaturation (22). Conversely, the relative efficiency of vitamin E should decrease with the level of unsaturation of the fatty acid (23). Two of the deficiency diseases, i.e. chicken encephalomalacia and muscular dystrophy in several species, violate these predictions. Nutritional encephalomalacia is produced by a C18:2n-6-containing vitamin E-deficient diet, but is reduced or prevented by substituting stripped C18:3n-3-rich oils or purified C18:3n-3 esters (24–28) or oils containing C22:6n-3 or its purified esters (25, 27, 29, 30). The onset of encephalomalacia is correlated with a relative increase in C18:2n-6 and C20:4n-6 (the latter fatty acid being greatly decreased by feeding stripped C18:3n-3-rich oils such as linseed oil (31)) in the cerebellum, which is the main tissue affected (32). The cerebellum has a high rate of vitamin E turnover (33) and consequently shows the largest decline in this vitamin during deficiency (34). The possibility that the protective effect of n-3 polyunsaturated fatty acids is via inhibition of C20:4n-6 derived thromboxane (TxB_2) has been ruled out since aspirin treatment, although drastically reducing TxB_2 levels, did not alter the incidence of nutritional chicken encephalomalacia (35).

Nutritional muscular dystrophy is another consequence of vitamin E deficiency in many species, particularly herbivora (first realized by Mason (36), for a review see ref. 37). In the rat, changes in muscle polyunsaturated fatty acids were not consistent with their susceptibility to peroxidation. C18:2n-6 was observed to decrease more rapidly than expected while C20:4n-6 was relatively increased in muscle phospholipids (38). In rabbits, muscular dystrophy has a reduced incidence when stripped C18:3n-3-rich oil is added to the diet (39). These observations have been confirmed by others in several species (22, 40–42). Selenium deficiency myopathy in lambs and calves has also been reduced by diets rich in C18:3n-3 (43). One report indicated that a small amount of cod liver oil (which is rich in C20:5n-3 and C22:6n-3) reduced muscle creatine kinase loss in vitamin E-deficient rabbits, whereas C20:4n-6 or stripped safflower oil increased the loss of this muscle enzyme (44). Similar observations have been made in the testis where a tocopherol-free diet containing low levels of C22:5n-3 and C22:6n-3 prevented sterility in rats (45). Probably C22:6n-3 could substitute for the normal C22:5n-6 found in rat testes, since it was incorporated in its phospholipids.

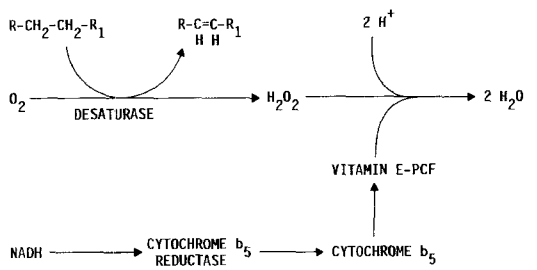
2.2. Spontaneous recovery from vitamin E and selenium deficiency

Other evidence inconsistent with the need for vitamin E as only an antioxidant is the original observation by Evans and Burr (46) that a significant fraction (17%) of the experimental rats on a vitamin E-deficient diet spontaneously recovered from the resulting muscular dystrophy without changing the diet; another 48% of the animals acquired a partial but not progressive paralysis. These observations were later confirmed by others in the rat (47) and in other species (48–50). Similar spontaneous recoveries have been observed with selenium deficient diets (51, 52). A genetic variability in susceptibility to vitamin E deficiency has been described in pigs (53). Coniglio, Whorton and Beckman (54) and Gabriel and Machlin (55) have bred a line of rats resistant to vitamin E deficiency. The possibility that this resistance could be explained by increased glutathione peroxidase activity is unlikely, since this enzyme activity is actually decreased by vitamin E deficiency (56, 57). Likewise, glutathione-S-transferase, catalase and superoxide dismutase are not increased by vitamin E deficiency (58).

The above spontaneous recoveries and resistance to vitamin E or selenium deficiency offer an extraordinary tool to elucidate the biological roles of these nutrients and to test the antioxidant hypothesis about them. However, it is disappointing that the value of these exceptions has not yet been exploited to understand or challenge the presently accepted roles of these nutrients.

3. Proposal for specific roles of vitamin E and selenium in the desaturation of essential fatty acids

In view of the above observations, I propose that chicken encephalomalacia and muscular dystrophy induced by vitamin E deficiency are due to a deficiency of the terminal desaturated-elongated products of essential fatty acids, particularly those of C18:3n-3 (i.e. C22:6n-3), created by an impaired synthesis of these highly unsaturated fatty acids due to this vitamin deficiency. I propose, therefore, that vitamin E (particularly RRR-alpha-tocopherol) participates in the endoplasmic reticulum electron transport involved in desaturation of n-6 and n-3 polyunsaturated fatty acids, as the terminal electron donor to the desaturase complex (Scheme 1). Alpha-tocopherol is known to undergo single electron oxido-reduction (59, 60). I further propose the existence of distinct and separate desaturases for each unsaturated fatty acid family, in which n-6 and n-3 desaturases compete for the terminal vitamin E-dependent electron transport factor with variable affinity. Within each desaturase family the desaturase classes acting on methylene groups nearest to the carboxyl group would have the least affinity for the vitamin E-containing electron carrier. Thus, in vitamin E deficiency the Δ4-desaturase would be inhibited the most within each fatty acid family. The n-3 desaturases, as a family, may have lower affinity for the vitamin E-containing factor than their n-6 counterparts (even though the n-3 desaturases seem to have a higher affinity for their acyl-CoA substrates than that of the n-6 desaturases). Therefore, under limiting vitamin E conditions, the desaturation of n-3 fatty acids would be preferentially impaired over that of their n-6 counterparts, as mutual depletion equilibria develop. This is consistent with fatty acid changes in nutritional encephalomalacia and muscular dystrophy discussed in Section 2.1. Thus, these nutritional diseases may be disorders of n-3 metabolism, induced by vitamin E deficiency, in these tissues rich in highly unsaturated n-3 fatty acids (viz. brain and muscle).



Scheme 1. Proposed desaturation mechanism for n-3 and n-6 polyunsaturated fatty acids. PCF denotes the proposed vitamin E-containing peroxidase coupling factor.

The respective desaturases are proposed to bind to the microsomal electron transport system through a terminal vitamin E-containing electron donor when loaded with their respective acyl substrates. This terminal electron donor is postulated to be associated with a selenium-containing peroxidase moiety; this complex may be loosely bound to the desaturase. It is therefore suggested that this terminal electron donor be called peroxidase coupling factor (PCF). RRR-alpha-tocopherol is proposed to have the highest binding constant for its PCF moiety in mammalian systems since this isomer is known (3) to have the highest biological activity. Because the PCF-peroxidase complex would contain Se, Se deficiency should produce changes in polyunsaturated fatty acid metabolism analogous to those elicited by vitamin E deficiency, i.e. n-3 desaturases should be more affected than their n-6 counterparts and Δ4-desaturation should be impaired the most. Fatty acid desaturation is proposed to be initiated by desaturase-generated $^1O_2$ and produce the 2 e^- product H_2O_2 via the intermediate $HO_2^{\cdot}$. The proposed peroxidase moiety removes H_2O_2 by a further 2 e^- reduction to two molecules of H_2O using electrons from the terminal vitamin E-containing electron donor and two protons from the medium (see Scheme 1).

Supplemental dietary selenium is known to delay the appearance of clinical signs of vitamin E deficiency (9). It is proposed that this supplement compensates for increased Se requirements caused by an increased turnover of the putative Se-containing peroxidase (and its associated desaturase). This in-

creased turnover would be due to enzyme inactivation produced by accumulation of H_2O_2 as a result of a decreased electron flow from the microsomal electron transport system due to a deficiency of its vitamin E-dependent PCF. In this way, vitamin E deficiency would produce a secondary Se deficiency. As vitamin E deficiency progressed, the inactivation of peroxidase would no longer be compensated for by its increased synthesis. This metabolic condition would thus initiate the clinical onset of vitamin E deficiency.

It is further postulated that the n-7 and n-9 families of desaturases and a proposed group of allelic or non-allelic n-6 and n-3 desaturases, which may be present in a low gene frequency, are vitamin E-independent enzymes. This is consistent with the observations that C18:1n-9 does not elicit encephalomalacia or muscular dystrophy in vitamin E-deficient animals (25, 26, 29), since these desaturases would not compete for the PCF. Individuals carrying such vitamin E-independent desaturases might activate the transcription of these enzymes under vitamin E deficiency conditions, allowing them to recover from the deficiency signs or to become resistant to the development of the resulting pathology. It is further hypothesized that the well known effects of some artificial antioxidants on the amelioration of vitamin E deficiency pathology may be due to their ability to substitute for vitamin E in the PCF. This would not be surprising since a number of artificial electron donors and acceptors have been found to substitute for their natural counterparts in the respiratory electron transport chain and in other oxido-reduction systems, including the Δ9-desaturase (61–64).

An alternative hypothesis for the effects of artificial free radical scavengers on vitamin E deficiency is that they may inhibit the secondary peroxidation induced by the series of secondary metabolic derangements which eventually leads to the accumulation of lipid peroxidation products, such as lipofuscin pigments. These derangements may include increased membrane turnover due to the aberrant fatty acid composition of its phospholipids (due to inhibition of desaturases) which in turn might elicit leakage of free radicals from membrane-bound oxido-reductase enzyme systems. These enzyme systems are known to be capable of initiating lipid peroxidation (65–67), and leakage of free radical intermediates and metabolites from some of these systems has been reported (68–70). Vitamin E-deficiency is known to elicit activation of the lysosomal system (71), which would be expected in order to handle an increased turnover of membrane phospholipids. Other metabolic conditions which may increase membrane phospholipid turnover, such as choline deficiency (72), are known to induce lipid peroxidation as well (73, 74). If artificial antioxidants were to act by inhibition of secondary peroxidation, they would be expected to only delay the appearance of the pathology, which might be somewhat different if was not complicated by secondary peroxidation.

4. Evidence in support of the proposed hypothesis tenets

4.1. Existence of different families of desaturases

In spite of the fact that different tissues (and within tissues, different cell types) in the same organism have a drastically different fatty acid composition (75–77) it is widely believed that Δ6-, Δ5- and Δ4-desaturases act on all the unsaturated fatty acid families; the acyl-specificity being thought to be achieved solely at the level of acyl-transferases. This notion originated from apparent competition for desaturases between the various unsaturated fatty acid families in feeding experiments (78, 79) and in *in vitro* experiments (80), and makes the unlikely implicit assumption that desaturation and acylation are coordinated poorly or not at all. This conclusion has been arrived at by ruling out other metabolic effects such as fatty acid activation and elongation on these desaturase competitions. However, possible competition of the various acyl-loaded desaturases for a shared component (viz. the terminal component of the electron transport chain) has not been considered in the published literature, even though enzyme competition for a shared component (such as a vitamin cofactor) is not uncommon in metabolic pathways.

Data are accumulating which strongly suggest that indeed there are specific desaturases for the various families of unsaturated fatty acids. The effect of feeding experiments with C18:2n-6 and C18:3n-3 in highly specialized organs such as brain and heart, which are known to normally contain high levels of C20:4n-6 and C22:6n-3, strongly sug-

gest that the Δ6- and Δ5-desaturations of n-3 and n-6 fatty acids are catalyzed by family-specific desaturases. Lamptey and Walker (81), in feeding experiments with safflower (n-3 poor) and soybean oils (n-3 rich), found that the latter oil did not cause lower levels of C20:4n-6, in brain phospholipids, than was obtained by safflower oil feeding in the controls; only C22:5n-6 was substituted for by C22:6n-3 in the soybean oil fed animals. These data show that n-3 fatty acids do not compete with their n-6 counterparts for Δ6- and Δ5-desaturation in this tissue. Similar results were reported by Kramer (82) in rat heart phospholipids, and by Crawford et al. (83) in voluntary muscle of several ruminant species. Stone et al. (84) and Huang et al. (85) reported analogous data in man and other species. These data together with the stringent conservation of C22:6n-3 in C18:3n-3 deficiency in various tissues (77) show that tissues such as brain, muscle and retina have a clear preference for n-3 fatty acids longer than 20 carbons.

Other observations consistent with the contention that n-3 and n-6 desaturases are independent enzymes come from temperature-dependent fatty acid changes obtained in fish. Acclimation of trout to lower water temperatures brings about a selective increase in the liver desaturation of n-3 fatty acids, particularly the Δ4-desaturation, i.e. synthesis of C22:6n-3, whereas the metabolism of n-6 fatty acids remains unchanged (86, 87). These data would not be expected if desaturations of n-3 and n-6 fatty acids were catalyzed by the same desaturases. A role of C22:6n-3-phosphatidylcholine in the sarcoplasmic reticulum (SR) Ca^{2+}-ATPase has been proposed (88). Thus the selective increased synthesis of C22:6n-3 in cold acclimated fish may not be solely for membrane fluidity purposes but to deliver it to muscle for an increased synthesis of Ca^{2+}-ATPase to maintain a normal rate of muscle contractility at lower temperatures (for a further discussion on the role of C22:6n-3 in the Ca^{2+}-ATPase see Section 5.1).

The apparent competition between n-3 and n-6 fatty acids *in vivo* is proposed to result from regulatory mechanisms and/or desaturase competition for the loosely bound peroxidase or PCF terminal electron donor or their complex. As proposed before, this competition would favour the n-6 desaturases as vitamin E-containing PCF becomes rate-limiting under vitamin E-deficiency.

Under the assumptions that the rates of fatty acid activation, elongation and acylation are not rate-limiting to the desaturation reaction, and that both n-6 and n-3 desaturase series have equal access to reducing reactants, and that dietary conditions are constant, one would expect that the mass action ratio of fatty acid substrates and products for each bond-specific desaturation reaction should be similar for comparable n-3 and n-6 fatty acids if each bond-specific desaturation is catalyzed by the same enzyme. A corollary of this postulation is that the ratio of n-6 to n-3 products for the same bond-specific desaturation (C20:4n-6/C20:5n-3 for Δ5-desaturation, C22:5n-6/C22:6n-3 for Δ4-desaturation, etc.) should not change under the same dietary conditions. However, this has been shown not to be true.

Developmental fatty acid changes in the mammalian brain have shown that the grey matter, in particular, drastically shifts from n-6 to n-3 fatty acids. The mass action ratio for the n-3Δ4-desaturase (C22:6n-3/C22:5n-3) drastically increases whereas that of the n-6Δ4-desaturase (C22:5n-6/C22:4n-6) is greatly decreased in the human brain (76). Similar changes were observed in the rat which were independent of the diet (89, 90). Although this *in vivo* data might be explained on the basis of a yet unknown developmental selectivity of the blood-brain barrier, this can be ruled out from data from brain cell cultures. When these cells are cultured under C18:3n-3 deficiency, neurons show a dramatic reduction in C22:6n-3 which is not compensated for by a corresponding increase in its n-6 analog, i.e. C22:5n-6; however the other cell types such as oligodendrocytes and astrocytes produce a compensatory increase in C22:5n-6 (91).

Developmental changes in the fatty acid composition of rat testes also show drastic changes in fatty acid composition upon sexual maturation. Rat testes show a 4-fold increase in C22:5n-6 between one to seven months of age, while the level of C22:6n-3 remains unchanged in the same period of time under constant dietary conditions (92). If a common desaturase was catalyzing the synthesis of both fatty acids, it would be unlikely to produce this difference. Rather, it is likely that these increased levels of C22:5n-6 are produced by an androgen-controlled translation of a specific n-6Δ4-desaturase.

In Menkes syndrome, a sex-linked recessive disor-

der of hypocupraemia which produces focal cerebral and cerebellar degeneration (93), a drastic decrease in the level of C22:6n-3 in the neuron-rich matter occurs (94). Consistent with the data of Bourre et al. (91) in neuron fatty acid changes upon n-3 fatty acid deficiency, the decrease in C22:6n-3 in Menkes syndrome is not compensated for by its n-6 analog. Rather, this n-6 fatty acid remains constant; a decrease of it should be expected if the same Δ4-desaturase is catalyzing the synthesis of both fatty acids. An impairment of the n-3 specific Δ4-desaturase may be involved in this disease if this desaturase requires copper for activity. A similar aberrant fatty acid composition is observed in the cerebral cortex in cases of infantile progressive encephalopathy (95–96) and lipofuscinosis in sheep (97). In both conditions, the n-3 fatty acids are decreased, whereas those of the n-6 series are increased or not affected. These data are consistent with a selective impairement of n-3 desaturases, particularly the n-3Δ4-desaturase, as the etiological factor in these disorders. The etiological factor in these related disorders may be a mutation in the gene coding for the proposed PCF. This would produce a functional vitamin E deficiency condition in its postulated role in fatty acid desaturation. This would be irrespective of the plasma levels of this vitamin, which are higher in the affected individuals (98). As proposed earlier (see Section 3) the accumulation of lipofuscin in both of these disorders may be an indirect consequence of impaired desaturases which would induce an increased turnover of membrane fatty acids (in this case, of the n-6 series due to a lack of the n-3 counterparts); this would overload the lysosomal catabolic capacity. Consistent with this contention is the recent observation that general lysosomal inhibitors produce an accumulation of lipofuscin-like pigments in rat brain (99). If defective PCF is the cause of these disorders, one therapy might be administration of the desaturated-elongated n-3 products.

In cystic fibrosis (CF), a recessive Mendelian childhood disease of unknown etiology, numerous fatty acid abnormalities are observed in plasma phospholipids. Lloyd-Still et al. (100) have reported unusual patterns of unsaturated fatty acids in CF children. These authors acknowledge that malabsorption cannot explain their data. An analysis of the reported unsaturated fatty acid levels by the mass action ratio criterion proposed above shows that these data are inconsistent with common bond-specific desaturases for both n-3 and n-6 fatty acids, as opposite mass action ratios are observed for Δ5- and Δ4-desaturases between both fatty acid series.

Other evidence which strongly suggests the existence of different desaturases for each family of unsaturated fatty acids comes from studies in carnivores. The cat, as an adaptation to its carnivore status, has lost both n-6 and n-3Δ6-desaturases and most of, at least, its n-6Δ5-desaturase (101, 102). However when this species is subjected to a diet containing saturated fatty acids as the only source of fat, the cat can induce the synthesis of 20:3n-9 (102). However, since this carnivore does not have the alternative Δ8-desaturation activity (103), the synthesis of 20:3n-9 must be catalyzed by an n-9 specific Δ6-desaturase, and most likely with an n-9Δ5-desaturase as well.

Other lines of evidence for distinct n-3 and n-6 desaturases come from cell culture studies. Maeda et al. (104) observed a loss of Δ6-, Δ5- and Δ4-desaturase activity for either the n-3 or n-6 fatty acid substrates in several cell lines and thus concluded that these desaturases for each series could be different. Spector, Mathur, Kaduce and Hyman (105) also observed that human 1603 skin fibroblasts displayed Δ6- and Δ5-desaturase activities with n-3 fatty acids, but not with those of the n-6 series. These results are consistent with similar data in a line of hepatoma cells. In these cells, Gaspar et al. (106) observed very weak Δ6-desaturation of C18:2n-6 but very high activity on C18:3n-3.

The proposed hypothesis of specific desaturases for each fatty acid series and their variable dependence on vitamin E could be tested kinetically. Substrate competition kinetics with varying levels of vitamin E should produce variable app.Km/app.Ki ratios for the competing acyl-CoA's. Another experiment would be to assay a desaturase, e.g. muscle Δ4-desaturase from tissues of C18:3n-3-deficient animals, with an n-6 fatty acid (C22:4n-6 in this case), using tissue from C18:3n-3 fed animals as controls. A similar experiment could be performed with C18:2n-6-deficient animals. A third possibility would be direct assay for a desaturase purified by affinity chromatography with an n-6 or n-3 fatty acid using the fatty acid analog not used in the purification. An experimental protocol for the purification of vitamin E-independent desaturases is suggested in section 5.2.

4.2. Participation of vitamin E and selenium in n-6 and n-3 fatty acid desaturation

4.2.1. Changes in polyunsaturated fatty acid metabolism

Infertility is one of several conditions resulting from vitamin E deficiency. In rat testes, this deficiency produces a decrease in the concentration of phospholipids to nearly half the normal value; a sharp decrease in relative % of C22:5n-6 and an elevation of the upstream intermediates (C22:4n-6, C20:4n-6 and C18:2n-6) is also produced (107, 108). Calculation of the mass action ratio of the n-6Δ4-desaturase from the above data shows that it is greatly decreased, indicating that this desaturase is greatly inhibited by vitamin E deficiency. The above fatty acid changes were later confirmed by others (54, 92, 109). An increase in the relative concentration of C20:4n-6 has consistently been observed in several other vitamin E-deficient tissues, such as liver and muscle (38, 45, 110–112). Selenium deficiency has also been shown to induce fatty acid changes similar to those of vitamin E deficiency. For example, voluntary muscle of selenium deficient lambs also shows a higher relative level of C20:4n-6 and a lower level of C22:6n-3 (113).

Under the proposed hypothesis, a greater inhibition of the Δ4-desaturase than the Δ6- and Δ5-desaturases in vitamin E deficiency would produce a transient accumulation of C22:4n-6 in the rat testis. The near equilibrium condition of the C20:4n-6-elongase would then produce a concomitant accumulation of C20:4n-6. In addition, the C20:4n-6-elongase may be increased by the vitamin E deficiency as a fallacious compensatory response (FCR) (an FCR is a compensatory response to a metabolic impairment based on a false assumption of a pathway control system about the location of the metabolic lesion; this response will normally result in a further accumulation of intermediates upstream of the lesion). Retroconversion of C22:4n-6 to C20:4n-6 is known to be near equilibrium (114–116). The preferential retroconversion of C22:4n-6 to C20:4n-6 in feeding studies (114, 116) suggests that the equilibrium constant of C20:4n-6 elongation favours the back reaction. The low levels of the Δ6-desaturation products C18:3n-6 and C18:4n-3 present in tissues is expected from the much higher activity of the downstream elongases (116, 117). However, since the the desaturases upstream of C20:4n-6 would also be inhibited in vitamin E deficiency (although to a lesser extent, according to the proposed hypothesis), the relative accumulation of C20:4n-6 must be transient. Since progress of the desaturase deficiency would not allow the establishment of a new steady-state condition, the relative accumulation of arachidonate is expected to be temporary, followed by the observed decrease in this fatty acid. This transient accumulation of C20:4n-6 has been experimentally observed (112, 118). Since phospholipids are required for C18:0 and C18:2n-6 desaturation (119, 120) it is likely that they are also required for other desaturases as well. If phospholipids containing the n-6 and/or n-3 desaturase products are part of these desaturase requirements, a negative feed-back loop may be established as the vitamin E deficiency progresses. This process may further decrease these desaturase activities and accelerate the progress of the pathology. A similar negative feed-back loop seems to operate in murine muscular dystrophy (121, 122).

Evidence consistent with the n-3 family of desaturases being more affected by vitamin E deficiency than those acting on the n-6 fatty acid series is abundant. Testicular fatty acid patterns from the vitamin E-deficient pig show a large decrease in the n-3 fatty acid series, particularly C20:5n-3, C22:5n-3 and C22:6n-3, while C20:4n-6 and C22:4n-6 accumulate (109). Similar results were observed in chicken testes (123), rat voluntary muscle (38, 118) and retinal pigment epithelium (124). Lower levels of C22:6n-3 and relative accumulation of n-6 fatty acids are also observed in muscle of Se-deficient lambs (113). In rat brain little change in the n-6 fatty acid series is produced by vitamin E deficiency, whereas C22:6n-3 is drastically reduced by this deficiency (89). Similar changes were produced in chicken brain phospholipids by vitamin E deficiency (32). In rabbit kidney and liver, vitamin E deficiency elicits a drastic reduction in C20:5n-3 while C20:4n-6 remains unchanged (125). These data are consistent with the postulation that the n-3 desaturases, particularly Δ4-desaturase, have a lower binding constant for the proposed vitamin E-dependent PCF.

Evidence consistent with the proposal that the n-9 desaturases are vitamin E independent comes from the fact that C18:1n-9, C20:3n-9 and C22:3n-9 accumulate in vitamin E deficient tissues (109, 118). Independence from vitamin E of the

C18:0-CoA desaturase is also suggested from the activity of the purified enzyme (119). An increase in liver C16:1n-7 from vitamin E-deficient ducklings (11) also suggests that at least the Δ7-desaturase is also vitamin E-independent.

4.2.2. Linoleate and acetate tracer studies

As discussed in the previous section, vitamin E deficiency in the rat produces sterility which is accompanied by a drastic decrease in testicular C22:5n-6 and relative increases in the precursors C22:4n-6 and C20:4n-6 (107). The hypothesis that these data may arise from inhibition of C22:5n-6 synthesis by vitamin E deficiency was investigated by Carney and Walker (126) with *in vivo* pulse labeling with testicular [^{14}C]-linoleate injection. These authors, however, only made a single time measurement, i.e. 24-hours after injection. At this time, label incorporation and radiospecific activities of C22:5n-6 and its upstream intermediates were higher in the vitamin E-deficient animals than in the controls. Misinterpretation of these data led the authors to conclude that no inhibition of C22:5n-6 synthesis was occurring in the vitamin E-deficient animals. It should be clear that a single time-point determination of label incorporation or radiospecific activity in this type of experiment has no simple direct relationship to the biosynthetic flux examined when the two treatments are expected to result in different turnover rates of the metabolites involved. If the vitamin E-deficient animals have an impaired flux of C22:5n-6 synthesis due to a lower activity of the enzymes involved, the lower expected fractional turnover rate will necessarily produce a slower transit of the label through the pathway. This situation can clearly be seen in a similar experiment reported by Coniglio et al. (54). These authors followed the label incorporation from an intratesticular injection of [1−^{14}C]-arachidonate into C22:4n-6 and C22:5n-6, between 4−72 h. At four h, the C22:4n-6 pool of vitamin E-deficient testis had almost half the radioactivity of that of the control; at 8 h this difference was abolished, whereas at 72 h the deficient animals retained more label than the vitamin E-supplemented controls. A similar pattern was obtained for label incorporation in testicular C22:5n-6 as well. If biosynthetic fluxes are calculated from the data of Carney and Walker (126), according to the equation derived by Zilversmit et al. (127) and assuming similar residual specific activity slopes for both treatments, it can be shown that in the vitamin E-deficient testes n-6Δ5-desaturase activity is about 55% that of the controls while n-6Δ4-desaturase activity is about 37% that of the controls. The rate of C20:4n-6 elongation, however, is increased by about 2/3. These data are consistent with the fundamental tenets of the proposed hypothesis, namely that vitamin E participates in fatty acid desaturation and that the Δ4-desaturase has a lower binding constant (affinity) for the vitamin E-dependent factor than the other upstream desaturases. This differential inhibition of desaturases plus the activation of the C20:4n-6-elongase (probably a fallacious compensatory response) can easily explain the transient relative accumulation of C20:4n-6 as discussed previously (section 4.2.1). An analogous activation of C18:3n-6 elongation can also explain the increased [^{14}C]acetate incorporation into C20:4n-6 observed by several workers (38, 45, 110, 128) in several vitamin E-deficient tissues. Increased acetate label incorporation has also been observed in muscle phospholipids of Se-deficient lambs (113).

Giasuddin and Diplock (20) developed a valuable tissue culture system as a tool to study the biochemical consequences of vitamin E, selenium and essential fatty acid deficiencies. This tissue culture was used to study the uptake of [^{14}C]linoleate by fibroblasts under vitamin E deficiency (21). Cells were incubated continuously with labeled C18:2n-6 for 48 h before harvest. The deficient cells showed an increased isotope incorporation in the C18:3n-6 elongase reactants, i.e. C18:3n-6 and C20:3n-6, whereas C20:4n-6 showed a drastic decrease in label incorporation. Butylated hydroxyanisole (BHT) could not abolish these effects of vitamin E deficiency. The authors concluded that these data could not be explained by vitamin E being only a non-specific antioxidant.

If desaturation rates for Δ6- and Δ5-desaturases are calculated from Giasuddin and Diplock's (21) data it can be shown that the n-6Δ6-desaturase activity in the vitamin E-deficient culture is about 70% that of the controls while n-6Δ5-desaturase activity is about 35% that of the controls. These rates together with those calculated from Carney and Walker's paper indicate that vitamin E (in this case the d-alpha isomer) is involved in desaturation of n-6 fatty acids and that the affinity of the bond-

specific desaturases for the proposed PCF-peroxidase complex is in the order $\Delta6 > \Delta5 > \Delta4$. The difference in binding constants among the various desaturases need not be large to account for these calculated differential activities. For instance, computer simulation studies with mutual depletion equilibria, in which vitamin E is depleted to 20% of its initial concentration, show that, if one assumes the ratio of n-6Δ6-/n-6Δ5-desaturase concentrations is 0.5, and enzyme concentrations are 10% and 20% of the initial vitamin E concentration, respectively, the above rates calculated from Giasuddin and Diplock's (21) data can be predicted, assuming the K_{diss}'s for PCF-peroxidase complex are 0.05 mM and 0.10 mM for the n-6Δ6- and n-6Δ5-desaturases, respectively (Infante, unpublished). The above assumed binding constants were chosen because they are similar to those of many enzymes for their co-factors.

The incorporation of linoleate label into phospholipids but not diglycerides observed by Giasuddin and Diplock (21) is expected from the operation of the newly discovered acyl-specific pathways of phospholipid synthesis (121, 122, 129). These pathways do not utilize diglycerides as acyl intermediates but use single fatty acid donors to acylate obligate glycerophosphodiester intermediates. This preferential incorporation of desaturated and elongated metabolites of C18:2n-6 into various phospholipid classes has also been observed by others (130–133). These data strongly suggest that fatty acid desaturation-elongation pathways are highly coupled to the glycerophosphodiester-dependent pathways.

4.3. Peroxidase activity of desaturase complexes

For several years it has been known that a high speed supernatant lipoprotein-like component activates various desaturases (134, 135). This activating factor, which may be loosely bound to the endoplasmic reticulum, does not seem to be NADH-cytochrome b_5-reductase, cytochrome b_5 or the terminal desaturase (134). Catala et al. (134) have shown that the n-3Δ6-desaturase undergoes a higher activation by the supernatant factor than the n-6 counterpart. This is consistent with the putative lower affinity of the n-3 desaturases for the PCF-peroxidase complex (discussed in Section 3). Baker et al. (136) have shown that catalase is one of the stimulatory proteins of Δ9-desaturase. However, this desaturation is the least responsive to the supernatant factor; this may be because this desaturase is non PCF-dependent and may lose less of its peroxidase moiety since it seems to be buried more deeply in the microsomal membrane (120, 135). Δ6-desaturation of C18:2n-6 was reported to be inhibited by exogenous H_2O_2, which was partially eliminated by addition of catalase (137). However, activation by the 'soluble' factor cannot be accounted for by its catalase activity alone (135). Catalase has also been noted to stimulate Δ15-desaturase in plants (138).

Sreekrishna and Joshi (139) have shown that divalent copper and its chelates inhibit C18:0 desaturation; since other free radical scavengers also inhibit this desaturase, these authors have suggested that superoxide radical ($O_2^{-\cdot}$) may be one of the active O_2 forms in the desaturase reaction. A similar involvement of $O_2^{-\cdot}$ in desaturation has been proposed by Holloway (140). These proposals postulate these oxygen radicals as the oxidizing species for fatty acid desaturation. However, the above reports are also consistent with the above desaturation scheme (Scheme 1) in which an activated form of O_2 (possibly $^1O_2$), generated by the terminal desaturase, withdraws two electrons and associated protons from the fatty acid forming the strong oxidant $HO_2^{\cdot}$ and finally H_2O_2. However, the HO_2 intermediate may be enzyme-bound and thus may not be readily available to the bulk medium (as its conjugate base $O_2^{-\cdot}$, since its pK_a is near 4.8) since superoxide dismutase has been shown not to affect the desaturation reaction (137). However, the inhibition of desaturation by low molecular weight free radical scavengers suggests they may be able to reach the active site, or they may act as electron sinks. In the proposed desaturase mechanism, the resulting hydrogen peroxide undergoes a further 2 e^- reduction from the terminal component of the microsomal electron transport system forming two molecules of water. A similar involvement of superoxide radical and hydrogen peroxide as intermediates in the 4 e^- reduction of O_2 by mitochondrial cytochrome oxidase has recently been proposed by Mitchell et al. (141). The substantial activity of the purified Δ6-desaturase observed by Okayasu et al. (120) in the absence of NADH supports the above suggestion, namely that the electron flow from the microsomal electron transport

system is not directly involved in the desaturation reaction but is necessary for the removal of its reactive product (H_2O_2) by further 2 e^- reduction to 2 H_2O. A further increase in activity produced by addition of catalase in the absence of reducing power would support this proposed mechanism. The striking decline of activity of the reconstituted Δ6-desaturase observed by Okayasu et al. (120) after five min of incubation is also consistent with the above proposed mechanism, since in the absence of PCF a poorly coupled electron transport system would allow the accumulation of H_2O_2 (if it is released to the bulk solution, addition of catalase might relieve this decline in activity). A similar inhibition by H_2O_2 may also occur in vitamin E deficiency. A possible interaction of the peroxidase moiety with cytochrome b_5 may explain the cytochrome-b_5 oxidation by H_2O_2 observed by Sies and Grosskopf (142) *in vitro*. An alternative proposal for this decline in desaturase activity is that, due to the purification protocol, the resulting desaturase is a PCF-independent isozyme (for further elaboration of this suggestion, see Section 5.2); the known phosphatidylcholine (PC) requirements of this desaturase may be for proper coupling to the electron transport system. If this is so, the acyl-specific PC species may be necessary for this function. Its reconstitution with egg PC liposomes may thus result in poor coupling since its acyl-composition is quite different from that of endoplasmic reticulum (ER) PC. If this is the case, reconstitution with ER PC should relieve the observed decline in activity.

5. Some corollaries of the proposed hypothesis

5.1. Role of vitamin E in the sarcoplasmic reticulum calcium pump

Highly unsaturated phosphatidylcholines, i.e. C22:6n-3PC, have been proposed to play a fundamental role in the coupling of sarcoplasmic reticulum (SR) Ca^{2+}-ATPase to Ca^{2+} transport, with an impaired synthesis of these PC species, at the level of the acyl-acceptor (glycerophosphorylcholine), proposed to be the primary lesion in Duchenne and murine muscular dystrophies (88). Experimental evidence in support of this latter proposal has been published (121, 122). According to the above proposed hypothesis on the role of vitamin E in polyunsaturated fatty acid desaturation (Section 3), a necessary corollary is that a deficiency of this vitamin should also elicit an uncoupling of the Ca^{2+}-ATPase from Ca^{2+} transport. This uncoupling due to vitamin E deficiency should induce the same sequence of secondary effects already proposed to lead to the observed muscle necrosis of hereditary muscular dystrophies (88). Such an uncoupling would be expected in the initial stages of vitamin E deficiency and would elicit a transient increase in the Ca^{2+}-ATPase half reaction along with a lower rate of Ca^{2+} transport activity, as is observed in some of the genetic muscular dystrophies (88). In addition to uncoupling, a fallacious compensatory response (FCR) may increase the net rate of synthesis of Ca^{2+}-ATPase as vitamin E deficiency progresses. However, the proposed induced secondary peroxidation (Section 3) would eventually result in inactivation of Ca^{2+}-ATPase thus counteracting its increased synthesis. This process would ultimately produce a net decrease of Ca^{2+}-ATPase activity in the latter stages of vitamin E deficiency.

Evidence consistent with the above corollary has been obtained in the rabbit. At earlier stages of vitamin E deficiency an increased Ca^{2+}-ATPase activity is observed (143), whereas at later stages of this deficiency a net decrease of this ATPase occurs (144–146) with a concomitant decrease in Ca^{2+} transport (145). Inhibition of both Ca^{2+}-ATPase and Ca^{2+} transport activities has also been observed with *in vitro* peroxidation of SR (147). Similar observations have been reported for Duchenne and murine muscular dystrophies (for a review see ref. 88). Therefore, both vitamin E deficiency and the above genetic muscular dystrophies would produce myopathy via impairment of the synthesis of C22:6n-3-containing-PC; one by inhibition of C22:6n-3 synthesis, the other by impairment of the synthesis of the acyl-acceptor, i.e. glycerophosphorylcholine. In both cases the target would be the sarcoplasmic reticulum Ca^{2+} pump.

5.2. Vitamin E and selenium independent desaturation pathways

In Section 3 it was proposed that only the acyl-CoA n-3 and n-6 desaturases utilize a vitamin E-dependent microsomal electron transport system,

whereas the n-7 and n-9 desaturases would be vitamin E-independent (supporting evidence discussed in Section 4.2). A corollary of this premise is that individuals which show a spontaneous recovery from vitamin E deficiency and grow to adulthood under these conditions (see Section 2.2) must have allelic or non-allelic vitamin E-independent Δ6-, Δ5- and Δ4-desaturases. Similarly, the spontaneous recovery from Se deficiency (see Section 2.2) may be due to the expression of a Se-independent peroxidase, probably a sulfur-containing enzyme.

The Δ6-desaturase purified by Okayasu et al. (120) may be one of the postulated vitamin E-independent enzymes. These authors purified the enzyme from livers of rats fed a fat-free diet. Such diets are known to induce vitamin E deficiency by impairing absorption of lipid soluble vitamins. The last step of their purification protocol included affinity chromotography to cytochrome b_5. This step would select against the putative PCF-dependent desaturases in favour of the vitamin E-independent isozymes (which are postulated to bind directly to cytochrome b_5). Consistent with this suggestion is the observed loss of about 80% of the activity in this final purification step. If this explanation is correct, the above purification protocol could be used to isolate the putative vitamin E-independent desaturases. These desaturases would likely be expressed in animals resistant to vitamin E deficiency while on these deficient diets. One would expect increased yields of enzyme when following the above affinity chromatography procedure with tissue from these animals.

Alternatively, individuals resistant to vitamin E deficiency may have the Δ8- and Δ10-desaturases (which could be vitamin E-independent) in addition to vitamin E-independent Δ4-desaturases. These operate on a desaturation-elongation pathway which is initiated by elongation. Evidence for a n-6Δ8-desaturase has been reported in rat testes (148) but not in liver (149). Desaturases acting on phospholipid-bound fatty acids (150) may perhaps also be vitamin E-independent. Tissues from those individuals which are resistant to vitamin E deficiency offer a most valuable but hitherto ignored tool to test this hypothesis and others on the biological role of vitamin E.

An analogous case of independence of an otherwise essential nutrient has been observed in some nitrogen fixing bacteria. Evidence for a non-allelic molybdenum-independent nitrogenase in *Azotobacter vinelandii*, proposed to be expressed under molybdenum starvation conditions, has recently been obtained (for a symposium report see ref. 151). Thus a similar situation for vitamin E may not be unlikely.

5.3. Biochemical diagnosis of vitamin E deficiency

From the hypothesis on the role of vitamin E in desaturation, one obvious prediction is that requirements of vitamin E for desaturation should be decreased if the required desaturated-elongated highly unsaturated products are provided in the diet. In this case, vitamin E or other antioxidants may only be needed for protection of these fatty acids during passage through the gastrointestinal tract and transport to the target tissues. The high susceptibility of herbivora (especially non-ruminants) to vitamin E deficiency (first realized by Mason, 1942) could be explained on the basis that these species have high requirements of this vitamin and of C18:3n-3 since their normal leafy diet contains only the precursor essential fatty acids, i.e. C18:2n-6 and C18:3n-3, with a particularly high level of C18:3n-3 (40–60% of total fatty acids). In addition, a leafy diet contains a high ratio of tocopherols to C18:3n-3 (152). Thus, herbivora may have a poor conservation system for vitamin E and C18:3n-3. The long gastrointestinal tracts of herbivores would normally make it difficult to provide the desaturated and elongated products of this fatty acid without extensive peroxidation. Therefore, the needs of vitamin E would not be a simple and direct function of dietary polyunsaturated fatty acids as usually believed.

Better indicators of vitamin E requirements may be proposed based on the postulated enzymatic function of this vitamin. One criterion for vitamin E deficiency would be an increased ratio of the elongated product of C18:2n-6 to its desaturated product, i.e. C20:2n-6/C18:3n-6 in phospholipids of vitamin E deficient tissues. This might be determined in cells with a high rate of turnover such as reticulocytes and lymphocytes, after feeding C18:2n-6. An increase in C20:2n-6 is observed in carnivore species fed linoleate, since the n-6Δ6-desaturase is low or absent in these species (101).

A second diagnostic assay for vitamin E defi-

ciency might be based on the mass action ratio of the acyl reactants of the Δ4-desaturases, especially that of the n-3 enzyme, i.e. C22:6n-3/C22:5n-3. As indicated in previous Sections, this desaturase appears to be the most affected by vitamin E deficiency, therefore a decrease in its mass action ratio may be a sensitive indicator for vitamin E deficiency.

A third assay for vitamin E deficiency may be based on the postulation that the n-3 desaturases are more affected by the deficiency than their n-6 counterparts. Therefore an increased ratio of the n-6/n-3 desaturase products for the same bond-specific desaturases, i.e. C20:4n-6/C20:5n-3 or C22:5n-6/C22:6n-3, should also be diagnostic of vitamin E deficiency.

It is hoped that these testable hypotheses will stimulate researchers in the field to re-examine the present inadequate hypothesis on the biological roles of vitamin E and selenium. It is possible that such research might shed light on the causes of some poorly understood diseases of polyunsaturated acid metabolism.

If glycerophosphodiester synthesis (e.g. glycerophosphorylcholine or glycerophosphorylethanolamine) is far-from-equilibrium (88, 129), accumulation of these species would be expected in vitamin E deficiency, as these are the putative acyl acceptors of the highly unsaturated fatty acids (129). In such case this accumulation could be a fourth diagnostic assay for vitamin E deficiency.

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